A Dose-Response Approach Differentiating Virulence of *Mycosphaerella fijiensis* Strains on Banana Leaves Uses Either Spores or Mycelia as Inocula

B.G.G. Donzelli[1] and A.C.L. Churchill[2,3]
[1] USDA-ARS, Biological Integrated Pest Management Research Unit, R.W. Holley Center for Agriculture and Health, Tower Road, Ithaca, NY, 14853, USA
[2] Department of Plant Pathology, Federal Plant, Soil and Nutrition Lab., Tower Road, Cornell University, Ithaca, NY, 14853, USA
[3] Current address: Department of Entomology, Comstock Hall, Garden Ave., Cornell University, Ithaca, NY, 14853, USA

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Abstract

The assessment of virulence of geographically and genetically diverse strains of *Mycosphaerella fijiensis* and resistance of their *Musa* hosts poses several challenges. *Mycosphaerella fijiensis* is slow growing and requires relatively strict culture conditions to produce conidia for inoculum. The size and relatively slow growth rate of banana plants from tissue culture further complicate screening efforts. In addition, relatively little is known about the effect of dose on disease outcome. A reliable and robust virulence assay using weighed fragmented mycelia brushed onto multiple defined areas (5 × 5 cm) of the abaxial surface of leaves of 3 to 6 month-old banana plants was developed. Methods to reduce the time needed for inoculum production and increase the number of assays that could be undertaken over time were developed. Image analysis software was used to measure the percentage of each inoculated leaf section showing black leaf streak symptoms, thereby allowing calculation and statistical analyses of leaf area affected using a dose-response model. The mycelium-based assay differentiated virulence levels of four *M. fijiensis* strains and revealed that one mutant, in addition to being deficient in conidia production and pigmentation, is of very low virulence. By localising inoculum onto small areas of large leaves, a dramatic increase in the number of strains that can be tested on each leaf and plant was achieved. The standardised method will be of value for evaluating *Mycosphaerella* pathogen virulence and *Musa* resistance where direct, quantitative measures during symptom development are necessary without requiring the use of expensive biotechnology-based tools.

INTRODUCTION

Measurements of virulence of foliar pathogens to assess differences in the ability of isolates to cause disease have been most often conducted by the application of conidia to plants at single inoculum doses followed by assessments of disease levels at single-time points using qualitative scales. Less frequently, measurements of virulence are conducted using the dose-response concept, where the effects on a plant caused by different doses of a pathogen are measured quantitatively. This latter approach is more robust than a single-dose assay and can be used with a significant degree of confidence and reliability to differentiate between strains with varying levels of virulence.

A method of banana leaf inoculation that bypassed the problem of inconsistent conidia production by *Mycosphaerella fijiensis* and increased virulence assay efficiency by applying inocula at multiple sites on large banana leaves was recently described (Donzelli and Churchill, 2007). The method also provided a way to quantitatively evaluate the virulence of four *M. fijiensis* strains by adoption of the dose-response concept. Data describing Area Under the Disease Progress Curve (AUDPC), assaying several concentrations of inoculum for each strain, were modelled using a General Linear Models (GLM) approach, and relative virulence levels were described by comparing the

values of the slopes and intercept lines estimated for each strain. The advantages of this approach included 1) the use of AUDPC data, which contained information about the intensity and timing of symptom appearance, and 2) the use of simple linear relationships to model doses of inoculum and AUDPC levels. While conclusive, this approach had the disadvantage of requiring two parameters (the intercept and slope of each linear equation) to define strain virulence. In the absence of a single numerical value with which strains can be compared, interpretation of the data can be challenging.

The key results of Donzelli and Churchill (2007) are summarised here, and a new analysis of the same dose-response data set to attain a simplified representation of the virulence levels of four *M. fijiensis* strains by estimating either their theoretical EC_{50}[1] or EC_{25} is reported. While the relative virulence levels reported are the same as in Donzelli and Churchill (2007), they are each represented by either EC_{50} or EC_{25} values, which are single quantities expressing the absolute estimated virulence level of each strain in our assay conditions. The dose-response approach is demonstrated as applicable to assays using either spores or mycelia as inocula.

MATERIALS AND METHODS

Organisms and culture conditions, inoculum production and plant inoculations, Colony Forming Unit (CFU) assessment, disease severity assessments and statistical analyses describing GLM and AUDPC using mycelial inocula were as reported in Donzelli and Churchill (2007). Briefly, *M. fijiensis* strains CIRAD743 and CIRAD301 (CIRAD, Montpellier, France) and spontaneous pigment mutants 743Pink and 301W2.2, derived from the respective CIRAD cultures, were used for plant inoculations. Mutant strain 743Pink is pink in colour and deficient in sporulation, whereas strain 301W2.2 has cream-coloured mycelium and sporulates abundantly. Mycelia were produced on DSM medium (Bradshaw et al., 2000) overlaid with cellophane and fragmented with a Mini-BeadBeater (Biospec Products, Inc., Bartlesville, OK, USA). Conidia of CIRAD743 were produced as previously described (Mourichon et al., 1987), suspended in 0.05% Silwet L77 (Loveland Industries Inc., Greeley, CO, USA), and the concentration was adjusted to $5x10^5$ spores/ml. Calibrated amounts of fragmented mycelium or spores were applied to 5 x 5 cm areas on the abaxial surfaces of 'Grande Naine' (AAA genome, Cavendish subgroup) leaves with a camel's hair brush.

All inoculations were conducted by randomly distributing inoculation sites on target plants (following a fully randomised design) using four (mycelium assay #1 and spore assay A) or three (mycelium assay #2 and spore assay B) replicates/experiment. Fragmented mycelia (60 µl) of four strains (743, 743Pink, 301 and 301W2.2) were used at 60, 12, 2.4 and 0.48 mg mycelium/ml. For conidial inoculations (spore assays A and B), 60 µl of spore suspension in 0.05% Silwet L77 at 5×10^5, 1×10^5, 2×10^4 and 4×10^3 spores/ml was applied. Inoculated plants were incubated in a controlled environment chamber as previously described (Donzelli and Churchill, 2007). Digital pictures of each inoculation point were taken weekly, and disease severity was evaluated with the image analysis software Assess 1.0 (APS Press, Saint Paul, MN, USA). Proportions of the inoculated area showing disease were arcsine-square-root transformed and used to calculate AUDPC by trapezoidal integration of disease severity over time for each treatment replicate (Campbell and Madden, 1990).

For GLM statistical analyses, both inoculum dose and AUDPC were log-transformed to linearise models and equalise variances, while the categorical variables (strain, plant and fragmentation treatment) were recoded as dummy variables. Data were further analysed by fitting disease scores after transformation with a Complementary Log-Log (CLL) link function, $CLL(y) = \alpha + \beta x$, where y is the proportion of disease, x = log (mg mycelium/ml) or log (no. spores/ml), and α and β are intercept and slope,

[1] EC_{50} is a synthetic parameter widely applied in fields, such as toxicology, invertebrate pathology and risk assessment, which represents the dose (i.e., estimated concentration) of an agonist (e.g., pathogen inoculum) needed to achieve 50% (or 25% for EC_{25}) of the maximal effect (e.g., disease severity).

respectively, of a linear equation. Disease scores used for this analysis were either disease severity at the endpoint of the assay or Relative AUDPC (RAUDPC) (Fry, 1978; Shaner and Finney, 1977). Normalisation of AUDPC scores was attempted using four alternative variables: 1) AUDPC from a theoretical dataset in which the disease level is 100% at the time of inoculation (Fry, 1978); 2) AUDPC from a theoretical dataset in which the disease level is 100% at the beginning of the necrotrophic phase of the disease; 3) AUDPC calculated using maximal disease severity within the analysed dataset; and 4) duration of the necrotrophic phase in days. Data were fit using the "all or nothing model #1" of Priprobit 1.63 (Sakuma, 1998).

RESULTS AND DISCUSSION

Delivery of fragmented mycelia onto discrete areas was effective in establishing black leaf streak disease (Fig. 1) (Donzelli and Churchill, 2007). GLM analyses allowed the modelling of AUDPC data from mycelium assays #1 and #2 as a function of strain, inoculum concentration and the interaction of strain and inoculum concentration. Mycelium concentration (mg mycelium/ml) was a more reliable predictor of disease levels as measured by AUDPC than CFU/ml (Donzelli and Churchill, 2007).

The use of discrete inoculation sites on leaves generated disease severity scores suitable for models used to analyse binary response variables, such as logit, probit and CLL probability functions. Differences in fittings among these possible choices were compared using Akaike's Information Criterion (AIC) (Akaike, 1974). With the dataset, the CLL link function performed consistently better than both logit and probit link functions (data not shown). Using CLL, final disease severity scores from mycelium assays #1 and #2 (Table 1, Fig. 2A and 2B) were modelled and theoretical EC_{50} values (Table 1) for the four strains analysed were calculated. Fitting of the endpoint disease severity scores from mycelium assay #1 did not pass the parallelism test, and each strain had to be fitted independently. Conversely, data from mycelium assay #2 passed the parallelism test. EC_{50} values from both assays were notably similar (Table 1) with the exception of those for 743Pink, which causes only a hypersensitive-like response in banana (Fig. 1). Strain 743 was the most virulent (Fig. 1 and Fig. 2A, 2B, 2D and 2E), with an EC_{50} of approximately 14-16 mg mycelium/ml. The EC_{50} values of strains 301 and 301W2.2 were relatively similar to each other and suggested they were about 5-8 times less virulent than strain 743. The EC_{50} values for 743Pink were notably higher than for all other strains in both assays, confirming its greatly reduced virulence. For 743Pink, the variability in EC_{50} values between assays and the large confidence limits were an artefact due to the overall poor growth of this strain in planta. Data for two independent inoculation assays, using spores of strain 743 (Table 1, Fig. 2C) were also modelled. The use of EC_{50} allowed the determination that the application of 60 µl of approximately 1.4×10^5 spores/ml of strain 743 as inoculum to a defined area causes roughly the same level of disease as 15 mg/ml of fragmented mycelium.

Since endpoint data do not provide any information about the dynamics of the infection over time, AUDPC was also used in an attempt to identify possible differences among strains. To use AUDPC with either probit, logit or CLL regressions, it was necessary to normalise the values and generate proportion data. Four approaches to normalisation were tested. Most approaches required assumptions about the values to be used as the reference in the normalisation and had the tendency to compress the range of data between the values of 0 and 0.6 (data not shown). This reduced both the performance of the analysis and the ability to detect differences among strains. Therefore, the duration of the necrotrophic phase in days, avoiding the aforementioned limitations, was used to normalise the data. EC_{25} rather than EC_{50} values were calculated to assess virulence of the strains, since EC_{25} falls approximately halfway in the response range generated by the normalisation.

Similar to the endpoint value analysis, we modelled RAUDPCs from mycelium assays #1 and #2 (Table 1, Fig. 2D and 2E) and spore assays A and B (Table 1, Fig. 2F), and calculated theoretical EC_{25} values (Table 1) for the four strains analysed. Data from

both mycelium assays passed the parallelism test, and EC_{25} values from both assays were relatively similar. As shown previously, strain 743 was the most virulent among the four strains, strains 301 and 301W2.2 were intermediate in virulence, and EC_{25} values for 743Pink indicated a negligible virulence level (Table 1) as observed in all previous analyses (Donzelli and Churchill, 2007). Using EC_{25} values and ratios, it was estimated that strain 743 is approximately 4-9 times more virulent than strains 301 and 301W2.2, and comparable to results seen when calculating EC_{50} values using endpoint data (Table 1). Similarly, the application of approximately 1.8×10^5 spores/ml (average of two spore assays in Table 1) of strain 743 to each inoculation site was estimated to be equivalent to using approximately 19 mg/ml of mycelium to attain comparable EC_{25} values.

CONCLUSIONS

The feasibility and convenience of using weighed, fragmented mycelia of *M. fijiensis* as inoculum for banana pathogenicity assays to detect differences in virulence of four strains on *Musa* spp. was recently demonstrated (Donzelli and Churchill, 2007). Furthermore, the utility and value of using quantitative AUDPC data and GLM analyses to rank strains for their levels of virulence was shown. Statistically significant differences in virulence were also identified among four *M. fijiensis* strains, one of which produces negligible numbers of conidia and, hence, could not otherwise be evaluated quantitatively for virulence using conidial inoculation methods (Donzelli and Churchill, 2007). Here, the statistical analyses used to quantify virulence levels, which included the use of the CLL probability link function to predict theoretical EC_{50} values calculated from endpoint disease severity scores and EC_{25} values from RAUDPC estimates, were further refined. Both approaches (i.e., endpoint and RAUDPC data) provided equivalent conclusions for this dataset regarding estimated levels of virulence. Further utilisation of such link function analyses by multiple laboratories will be necessary to determine if there is a single approach that should be recommended in the future as a general standard. Nevertheless, the approaches described here provide a first step towards assigning a single numerical value that can be used to quantify virulence of *M. fijiensis* strains rather than relying on the slope and intercept values provided by GLM analyses for such comparisons. The results also confirm the problem of variability that continues to hamper banana pathogenicity assays and points to the importance of identifying plant growth conditions that will reduce plant-to-plant and assay-to-assay variability. The methods described here will be of value for evaluating *Mycosphaerella* pathogen virulence, as well as *Musa* spp. susceptibility, in a broad range of settings where direct, quantitative measures over the full course of symptom development are necessary.

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Tables

Table 1. Equation coefficients and EC values estimated for mycelium assays #1 and #2 and spore assays A and B using either endpoint disease severity scores or RAUDPC.

Strain	Intercept			Slope			EC[c]		
	α	SE[a]	*P*[b]	β	SE[a]	*P*[b]	Value	UCL[d]	LCL[d]
Mycelium assay #1 – endpoint data[e]									
301	-3.56	0.18	0.000	1.59	0.12	0.000	103.1	55.1	362.6
301W2.2	-5.90	0.41	0.000	2.87	0.24	0.000	85.2	64.1	135.8
743	-2.85	0.12	0.000	2.17	0.09	0.000	13.7	9.7	18.8
743Pink	-12.77	5.49	0.020	4.72	3.13	2.282	423.4	ND[g]	ND[g]
Mycelium assay #2 – endpoint data[f]									
301	-5.44	0.47	0.000	2.46	0.25	0.000	117.0	71.7	208.7
301W2.2	-5.04	0.45	0.000	2.46	0.25	0.000	79.7	53.2	126.5
743	-3.29	0.37	0.000	2.46	0.25	0.000	15.6	11.3	20.7
743Pink	-9.86	2.17	0.000	2.46	0.25	0.000	7348.50	130.47	637,530
Spore assay A – endpoint data									
743	-8.93	1.40	0.000	1.67	0.27	0.000	130,560	78,523	211,330
Spore assay B – endpoint data									
743	-19.33	1.86	0.000	3.68	0.36	0.000	151,700	131,230	171,490
Mycelium assay #1 – RAUDPC data[f]									
301	-5.31	0.20	0.000	1.99	0.11	0.000	108.8	83.7	145.1
301W2.2	-5.60	0.21	0.000	1.99	0.11	0.000	152.5	112.0	214.8
743	-4.04	0.18	0.000	1.99	0.11	0.000	25.1	21.6	28.9
743Pink	-8.44	0.53	0.000	1.99	0.11	0.000	4045.5	1220.1	14971.0
Mycelium assay #2 – RAUDPC data[f]									
301	-5.94	0.41	0.000	2.26	0.20	0.000	118.9	70.6	215.6
301W2.2	-5.44	0.38	0.000	2.26	0.20	0.000	71.4	47.6	111.6
743	-3.79	0.33	0.000	2.26	0.20	0.000	13.4	9.8	17.1
743Pink	-9.78	1.68	0.000	2.26	0.20	0.000	5965.8	201.1	ND[g]
Spore assay A – RAUDPC data									
743	-10.05	1.48	0.000	1.65	0.28	0.000	214,690	134,340	347,410
Spore assay B – RAUDPC data									
743	-17.41	1.03	0.000	3.12	0.19	0.000	142,750	118,190	173,280

[a] SE - standard error.
[b] *P* - significance level.
[c] EC values reported are EC_{50} for endpoint data and EC_{25} for RAUDPC data. In both cases, they are expressed either as mg mycelium/ml (mycelium assays) or number of spores/ml (spore assays).
[d] Upper (UCL) or lower (LCL) 95% confidence limits.
[e] Since parallelism of the slopes was rejected, the dataset from each strain was fit independently from the other strains.
[f] Since parallelism of the slopes was not rejected, the dataset from all strains was fit using a single slope value.
[g] ND - not determined.

Figures

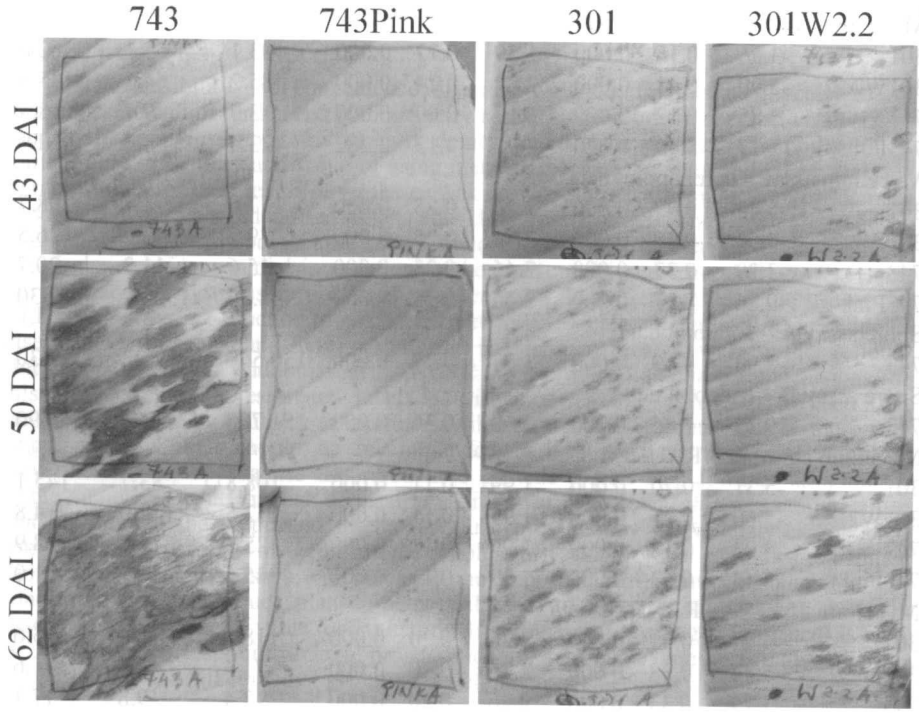

Fig. 1. Black leaf streak symptoms observed at the same inoculation sites 43, 50, and 62 days after inoculation (DAI) using 60 µl of 60 mg/ml fragmented mycelia from *Mycosphaerella fijiensis* strains 743, 743Pink, 301, and 301W2.2 as inocula in mycelium assay #1. Reprinted with permission from Donzelli, B.G.G. and Churchill, A.C.L. Phytopathology 97:916-929. ©2007 Phytopathology.

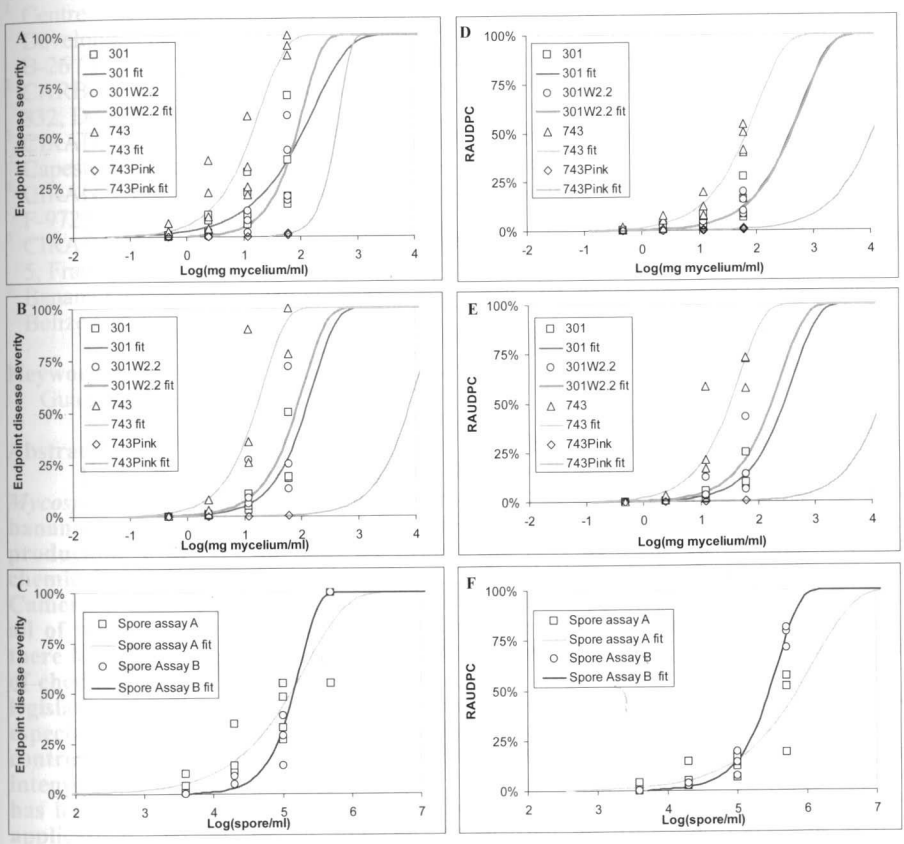

Fig. 2. Complementary Log-Log (CLL) fittings of endpoint disease severity scores (plots A, B and C) and RAUDPC (plots D, E and F) of mycelium assays #1 (A, D) and #2 (B, E), comparing *Mycosphaerella fijiensis* strains CIRAD301, 301W2.2, CIRAD743 and 743Pink, and spore assays A and B (C, F) using CIRAD743.